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Genes Encoding Components of the Olfactory Signal Transduction Cascade Contain a DNA Binding Site That May Direct Neuronal Expression

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Genes which mediate odorant signal transduction are expressed at high levels in neurons of the olfactory epithelium. The molecular mechanism governing the restricted expression of these genes likely involves tissue-specific DNA binding proteins which coordinately activate transcription through sequence-specific interactions with olfactory promoter regions. We have identified binding sites for the olfactory neuron-specific transcription factor, Olf-1, in the sequences surrounding the transcriptional initiation site of five olfactory neuron-specific genes. The Olf-1 binding sites described define the consensus sequence YTCCCYRGGGAR. In addition, we have identified a second binding site, the U site, in the olfactory cyclic nucleotide gated channel and type III cyclase promoters, which binds factors present in all tissues examined. These experiments support a model in which expression of Olf-1 in the sensory neurons coordinately activates a set of olfactory neuron-specific genes. Furthermore, expression of a subset of these genes may be modulated by additional binding factors.

The olfactory system is capable of detecting an extremely wide range of odorant stimuli with high sensitivity and selectivity. The initial signal transduction events in odorant detection occur in the sensory cilia of olfactory neurons (reviewed in reference 17). Several of the proteins in this signalling pathway have been identified and localized to the cilia by immunohistochemical and electrophysiological methods. These components include the putative odorant receptors, $G_{olf\alpha}$, type III adenylyl cyclase, and the olfactory cyclic nucleotide gated channel (OcNC). Significantly, each of these genes is expressed exclusively or predominantly in the olfactory system (2–4, 9, 10, 12, 13). Additionally, three olfactory neuron-specific cDNAs (olfactory marker protein [OMP], 50.06, and 50.11) have been identified by differential hybridization screening and other molecular genetic techniques, but the functions of the proteins encoded by these genes remain unknown (18, 19).

The olfactory receptor cells are the only neurons in the adult which undergo continual replacement. These cells have an average life span of 60 days and are functionally replaced from a population of neuroblast-like basal cells that undergo continual proliferation and differentiation (8). The morphological differentiation of basal cells into mature olfactory neurons parallels the induction of olfactory neuron-specific gene expression (20). These unique features of the olfactory system make it an ideal model for the study of transcriptional regulation during neuronal development. As a first step in understanding the molecular mechanisms which control the expression of genes which distinguish the mature neuronal phenotype, we have attempted to identify conserved *cis*-acting sites present in their promoter regions. The promoter regions of genes encoding five olfactory-specific proteins were isolated and analyzed. Each of these genes contained at least one site for the olfactory neuron-specific DNA binding activity, Olf-1. In addition, two genes which participate in odorant signal transduction contained an additional novel binding sequence, the U site, for a widely expressed sequence-specific DNA binding activity.

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MATERIALS AND METHODS

Isolation and analysis of genomic clones. Random prime labeled cDNA clones for $G_{olf\alpha}$(10), type III adenylyl cyclase(2), OcNC(4), 50.06 (18), and 50.11 (18) were used to probe 5×10^5 plaques of a rat genomic library (Stratagene) under high-stringency conditions (washes were performed at 65°C in 0.5 × SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate [SDS]) (14). Restriction fragments from each recombinant phage which hybridized with radioactive probes generated from the 5′ end of each of the cDNAs were subcloned into Bluescript KS (Stratagene). DNA sequencing was performed with Sequenase (U.S. Biochemical).

Primer extension analysis. Primer extension to determine the transcriptional start sites for each of the genes (1) was performed with the following oligonucleotides: MW39 ($G_{olf\alpha}$), 5′-TTCCGCGGTCTTGCTGCTGTTGCCCAAACA; MW40 (type III cyclase), 5′-GCTGCTTCCCGCAGTGC CGCCGCGCCAGCT; MW41 (50.06), 5′-CGGTCACCAAG GATACAGGAGCTCAGGGAT; and MW42 (OcNC), 5′-AGGCCCAATGGAGCACATAACACAGCTAGG. Oligonucleotides were ^{32}P end labeled by using T4 polynucleotide kinase and incubated with 5 μg of olfactory tissue total RNA in 120 mM KCl for 1 min at 100°C, 5 min at 85°C, and 30 min at 55°C. Extension reactions were performed by bringing the annealing reactions to 42°C and adding reverse transcription buffer (final concentrations, 20 mM Tris [pH 8.3 at 42°C], 10 mM dithiothreitol, 10 mM $MgCl_2$, 0.5 mM deoxynucleoside triphosphates, 125 μg of actinomycin D per ml, 50 mM KCl, 1 U of RNasin per μl, and 1 U of avian myeloblastosis virus reverse transcriptase per μl) in a final volume of 30 μl for 30 min. The extension reaction products were phenol ex-

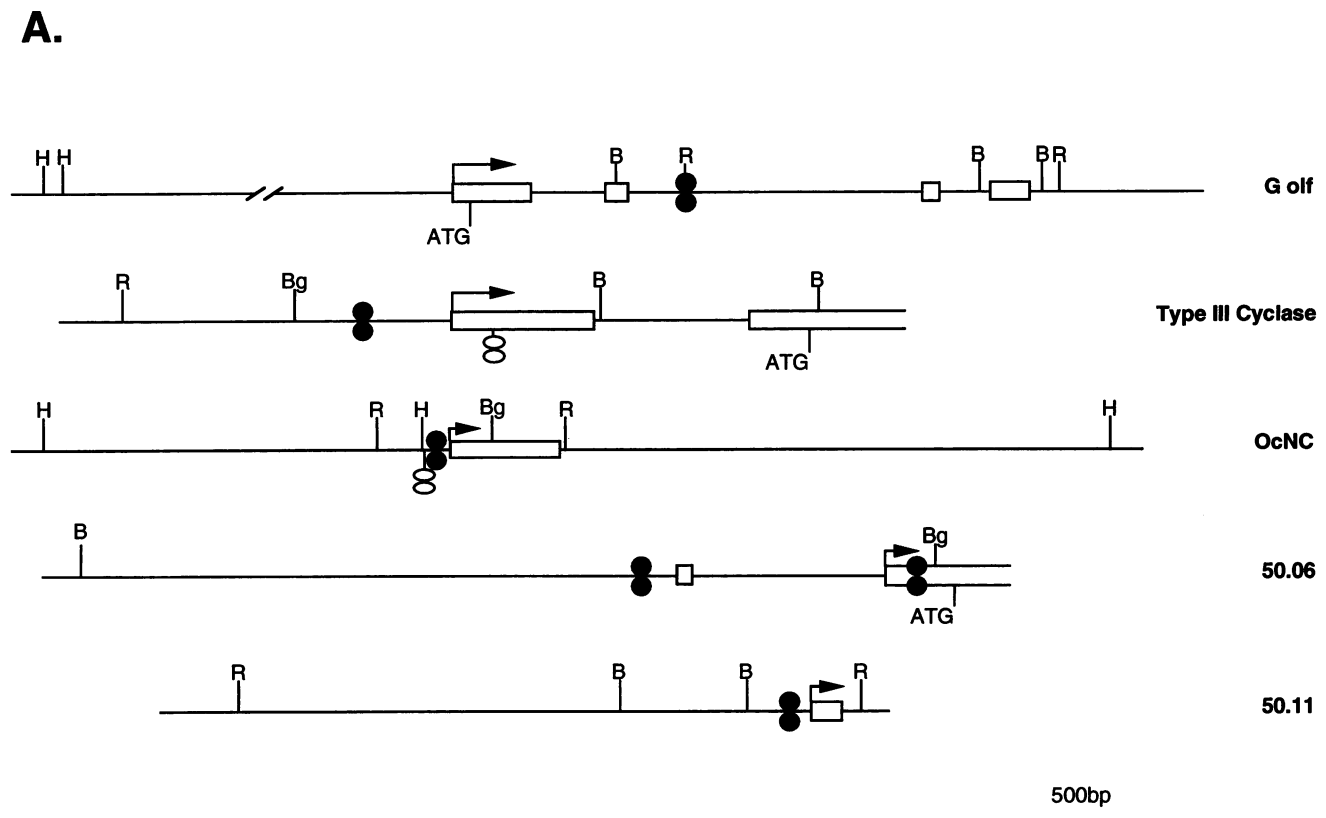


FIG. 1. Structure and promoter sequences of rat olfactory neuron-specific genes. (A) Restriction maps of $G_{olf\alpha}$, type III cyclase, OcNC, 50.06, and 50.11. Exons are represented by open boxes. Olf-1 and U sites are represented by solid and open circles, respectively. B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; R, *Eco*RI. The $G_{olf\alpha}$ gene has introns located 145, 220, 274, and 394 bases downstream of the initiating methionine (10). An intron in type III adenylyl cyclase occurs 179 nucleotides 5′ to the initiating methionine (2). An intron in OcNC occurs 18 bases before the initiating methionine (4). In 50.06, an intron is located 172 nucleotides before the initiating methionine (unpublished results). The 5′ *Hin*dIII-*Bam*HI fragment in $G_{olf\alpha}$ is 3 kb. Arrows and ATG indicate starts sites for transcription and for translation, respectively. (B) Sequences surrounding the start sites were determined and the locations of the start sites were identified by primer extension and analysis of cDNA clones extending to the 5′ end of the gene. Nucleotide sequences have been numbered so that nucleotide +1 represents the first nucleotide of the mRNA. Exon sequences are boldfaced. Two major start sites were identified for 50.06 at positions +1 and +4. The type III cyclase start site shown was determined by the size of the major product of the primer extension reaction. One cDNA clone for type III cyclase extended to position −82 of the sequence shown. (The start site for 50.11 was not determined by primer extension; the site indicated by the arrows represents the start of the longest cDNA sequenced.) The Olf-1 site for $G_{olf\alpha}$ is not included in these sequences. Olf-1 sites are boxed. Consensus U box sequences are doubly underlined in the type III cyclase and OcNC sequences.

tracted, ethanol precipitated, and sized on 6% polyacrylamide–urea sequencing gels.

Nuclear extract preparation and binding assays. Nuclear extracts were prepared from 3-week-old Sprague-Dawley rats essentially as described elsewhere (5) except that olfactory tissue was homogenized by using a Brinkmann polytron. Other tissues were Dounce homogenized. Radiolabeled fragments for footprinting the OcNC promoter were generated from plasmid p4.1RI500, containing nucleotides −312 to +154 of the OcNC gene cloned into the *Eco*RI site of Bluescript. The plasmid was cut with either *Xho*I (to detect binding on the top strand) or *Bam*HI (to examine binding on the bottom strand), treated with alkaline phosphatase, ^{32}P end labeled with T4 polynucleotide kinase to 10^4 cpm/ng, and cut with either *Bam*HI or *Xho*I to release a 0.5-kb labeled insert which was purified by agarose gel electrophoresis. Footprinting (1) was performed in a volume of 100 μl by incubation of 15 to 30 μg of protein and 100 pg of the probe in 6.5 mM Tris (pH 8.0)–3.25 mM $MgCl_2$–0.65 mM $CaCl_2$–1.3 mM dithiothreitol–63 mM KCl–32 μg of bovine serum albumin per ml–1.3 μg of salmon sperm DNA per ml–2% polyvinyl alcohol (molecular weight, 10,000). After a 15-min incubation at 25°C, DNase I was added, and the incubation was allowed to continue at room temperature for 2 min. Reactions were terminated by addition of an equal volume of stop buffer (200 mM NaCl, 20 mM EDTA, 1% SDS, and 50 μg of tRNA per ml) followed by phenol extraction and ethanol precipitation. Products were denatured at 95°C in the presence of 50% formamide and resolved on polyacrylamide-urea sequencing gels.

Gel shifts were performed essentially as described elsewhere (6, 7) with the following minor modifications. Probes were prepared by annealing a 10-fold excess of a complementary oligonucleotide to single-stranded oligonucleotides which were ^{32}P labeled by phosphorylation with T4 polynucleotide kinase. Binding reactions contained 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9 at 4°C), 10 mM $MgCl_2$, 10% glycerol, 50 mM KCl, 0.5 mM dithiothreitol, 100 μg of dI-dC per ml, 100 μg of salmon sperm DNA per ml, 5 to 15 μg of protein, and 200 pg of ^{32}P-labeled oligonucleotide probe (2,000 Ci/mmol). After a 10-min incubation on ice, the mixture was electrophoresed

B.

G olf

ATGTGACTGGTGAGCCTCCTAGAGATCCTCCTCCCTGATAGAATATGCGTGATCCGAGTCCTCACTCGCCTGGGAGCCTGGAGGCGTCGGAGCTGTGCGAGGAGCGCGTACCCATACGCACTGGGGCGCGGGGTGC -557

AAGAAAGCCACGCGGGTCAGCGTGTAAGCAAGCGCCCCTTGTCCCTCCTCTGACTCATCAGGTTAGGGAAACGCTCCTCTGAGCCCCCAAGCCAACCCCTACCCCAGTTCCCATCTCCTTCCAGGAGAGCGGGACC -421

CGCAAGGGGCGCGCACATCAGACAGCAAAGCAACGAGTCGGCCCACAGTCGCCTTGTTGGGACTCTTTGGTACGAACTCTGGCCCTTGGTGCTGTGCTTAGCCACTACGAGGGGCAGGACCCTGCTTTTGGTTGCG -285

CACCAACTCCCCCTCAGCATCTCCGTGGGGCGGTGCTGAGCCAGGAGCTGGCTGAGCCCGGGCACAGCGCCACCCCGCTGTCTCCAGCAGAACCCGGCGCCAGGGGGGTTCCGTGCGCTGTGCGCTGTTTCCCCAG -149

CAGGTTCCTAAGACGACCTGCCTTTAAGGAGCGTTTCCCTGGGCGCGCCTGCCTCCCGGCGCCGTTAGCCGGGAGCGTGCGGTGGGCAAGGGAGGGAGAAGCCGGTGCGATCGTAGATCCCCGGGAGACCTGGCCG -13

TCTCTTTCAGTATCAGACAGCGGAGAAGCCGTCGCGGGAGCGCACTTATCGGGGTCTCGCTGGCTCTGGCTCTGGTGCGCCTTCCTACTTGCCTGATCGGAGTGCGAGCCAGCAGGCATGGGGTGTTTGGGCAACA +124

GCAGCAAGACCGCGGAA +141

Type III Cyclase

CCCTATGGCTGTTCTTTCCTCATCTTGAGCTGCCTCCCAAAGTGATCCCTTGATGCTGAGGGCTCTTCCCAGTCCCCTGAGTACATTTTCTTCTGAAAACGACTTTTACTCCTAACAAGGTGTTCTGTCAAAATTG -455

ATACCACGGCTTAGCAGACTGATAGATTCAAAAGGAACATTTGCGTCTTTAAGGAAGAATCCAAACACATCTCAATTGGTAGGATGGAGATCTTCGAGAAGCAAGGCCTCTTTCGGGCCCACGTGCCAGGTACCAA -319

GCAAGCTACTCCAGGCCTCTTTTGTCACCAGGTTAGGCTGTATATTCCCTTGAGGAGAGATCAAGCCCCACGCTTGACGTCTGGGCTGCCAGGGTAGGCGGAGACACTGAGATGGGGCGCGCCCATTCGGGGAGCG -183

GTCCCCAAACCTCAGCCAGGGCCTCTCCTTTCCCCGGAAGGAAACCAGGGTAGAAGGGCGGCTGGGGTGCCTCAGCACGCCTCACCTGCAGGGAGGATACGTCCTGGGCTTTGATCTTCTGTAACACGGTCTGTTC -47

GAGGCAAAGCGTGGAGTCACCTCGCTCTTTTCTGTCTGCCACCTACTCGGCGTTTCCACGCAGCTATCTCCGGGCGCTGGCAAGTGGGGGGACACCGGGGACAGCGAGGGGCCCCTCCACCCGGCGTCCAGGAGGC +90

GGCGACAAGGCGGGGAGGGGCCGCCCGCCTCCTGGGGCCTGCGGCGCGCGTGAATCAGTGTGCAGAGCGCGGCGCACGGACCTCGGCTGGCTCGCCCTGCGTGCGGGGAGAGGATGCTGGCAGGCTGCCCCGGCCT +126

GGGCCCGCCATCCGCAGCGTGAGCCACCGCCGTCCTCTGCGACCACGGATGCTGAACCACAGCCCCGCGCCTCTACCGGCCCGACCCGGGGCCTGACCGCAAGGGGACCCGCGCACAGGCTCCGGGCGGAGGAGCT +362

GGCGCGGCGGCACTGCGGGAAGCAGC +388

OcNC

AGCCCTTGTAAAGAGCTAAATCCATTTTCTCCTCTCTGGCTGTTCTTTCTTTTCTCACCCAGATTTCTCCCTGATTTAGCCTCCAATAAGGTGGGCTGTCTGGAACTCTGCCAGCACTTTAAGGCAGTGGTTTGGT -119

GGCCATGAGCTTGCAGAAAGAAAGCTTTGTACCTGTCTGTGCAAGGGAGAGTCCCTAGGGAGCTTGGGAGGGGCCAGACAAGCAGCTTCCACAGACGGGCAGCCTAGTATCTAGTCCTCAGTTGTTGATAGCTACG +18

TGTGTGCACAACCACATTGAAGATGGCTTCTCCAGGGGCCGGGGCACCCTGATAGGCTCTCAAGCTCACCTAGCTGTGTTATGTGCTCCATTGGGCCTCTGTCAGCTGCTATCAGACAGCGTGGGCTAGATC +150

50.06

GATCCTATACCATCATACCAGGCCAAACTGTGGCTGAAAAGAGGTCATAGCTTGCTTCAATCCTAACTTCCTTTTCTACCCATCCATGAGGTCCTGTGTTTCTGTCCCTTTAAGTATCTCAGCCTGTTGCTAGGCT -97

CTCAGGATTCCCCAGGGAGGGGACCAGGTGCGTCACCTGTCACCTGTGTGCCTCTGGGACCCTAGTGCCTGTTCCTTCCAGCGCCATTGCCATTCGGCAGCACTCCCTGCCTACCTCTTTGTTGAGAGCTGATCAT +40

CTGCCCTTGTCTTGGACATCACTGAACAGGTAAGAGACCCCATGACTCCAAATGACCCTGTCCCCCCACCCCAAGCTCACTCCTCCATCTCCACTGCACCACAGGAGTTACTGGGCCCCTAGCTCCATCATCTGTA +176

AATGGGCCTGAGCCCGCTGTCCTGTCTGCATCCCCATCAGGCTGAGGAGTCCCACACCTCACATCCCTATTTCTTGAAGACCTGGAACTCTAAGATCCAGACATCATTGTCTAACCCGTGGTCCGTGATCCCATGTC +312

CTTTCCCCTGTAACCCTTTAGCCCTGCTTGTAGGCCCCAGTATTCAAAATCTCTTAAGTACCTAATTTCTGGCAAGAACCAAAGACAGAGACCCGTTGCCCTCCACTCTGTGGGACTCAGAGCCCACAGTTTGCTT +448

CTCAGGACTGTGGAGTCTTCCTCCTCCCTCTGACTGAGTTTGGGCCCTGGCTCTTCATTCTCATCCTCCTCAGGTTTTAAAGCCTTTGCAGTCCTGGCCTCCTAAGAACCTTGGGAGAAGGTTGTCCTATGTCCCC +584

TTGGCCATCTGACACCCTCTGAAGGTGGAGTCTAACATTTGTGGGACCTTAGAAAGGTGTCATGTCCCAGTTTTTCAGGGACCCCACTGGGAAACGTACTACCACTCCCAGGAATCCCTGAGCTCCTGTATCCTTG +720

GTGACCGGTTACCATCCCACAGCCTCCAGGGAGTTGCTGTTGGCCTTTGCCAGGGTCCCTGGACAGATC +789

50.11

GGATCCATGTGGCATATCTTACAGGAAGCAGCCTCTGGCAGAAGGCTCTTTTCTTTTGCCTTTTGCCTTTGAACAAAGACAGTGTGAAGCCAGGGAAGGTGCTTTGTTTATGTTTCTAGGCTCCTTGCACCAGGGT -91

TACTACTCCTCAGGGTATATGCCAAGGAGAGGGAGCCTCTCTTGCTCTCCACACGCACGCCACCGAGCTGGTGTCTGCTGCTGGGCTTGAATCCCGACCCAGGACCATCTGCGAGATTTGACAAACTGTCTCAAGA +46

ATGAGATGGATTCACCCGAGAAACTGGAGAAGAACAATTTAGTGG +91

FIG. 1—*Continued.*

on a 6% polyacrylamide gel (acrylamide/bisacrylamide ratio, 59:1) in 0.25× TBE (1× TBE is 90 mM Tris-borate [pH 8.3]–2 mM EDTA) at 4°C. Products were detected by autoradiography of the dried gel. The sequences of the oligonucleotides used in binding assays are displayed in Fig. 5C and 6C. Mixing experiments included 4 μg of olfactory tissue nuclear extracts and 10 to 15 μg of nonolfactory tissue nuclear extracts.

Competition experiments were performed by incubating binding reaction mixtures with unlabeled competitor DNA for 5 min before the addition of the probe. Oligonucleotide competitors were used at 7.5 ng/μl. Plasmid competitors

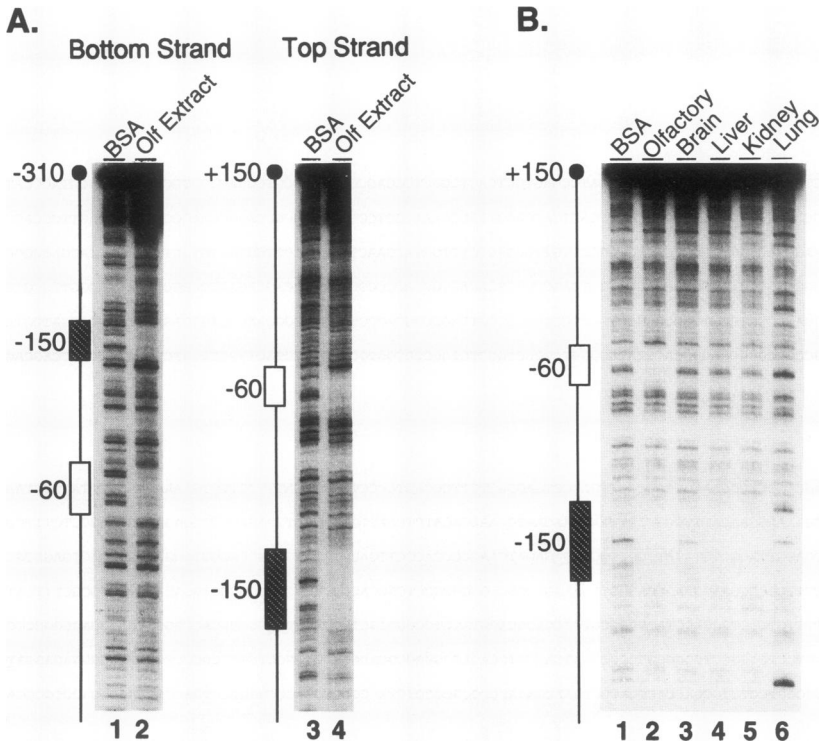


FIG. 2. Footprint analysis of the OcNC proximal region. (A) The bottom strand (lanes 1 and 2) or top strand (lanes 3 and 4) of the 5′ flanking region of OcNC was end labeled and subjected to limited DNase I digestion in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of olfactory nuclear extracts. Two regions of protection were seen: the OcNC U site (shaded boxes) and the OcNC Olf-1 site (open boxes). (B) The top strand probe was incubated with nuclear extracts from the indicated tissues and subjected to DNase I footprinting analysis. BSA, bovine serum albumin.

were added at a 100- to 250-fold molar excess over probe concentration. In plasmid competition experiments, plasmids which consistently blocked binding of Olf-1 protein compared with vector competitor were identified as Olf-1 binding.

RESULTS

Isolation of olfactory neuron-specific promoter regions. The putative promoter regions for each of the olfactory neuron-specific cDNAs were isolated from a rat genomic library by screening at high stringency with cDNA probes from $G_{olf\alpha}$, type III cyclase, OcNC, 50.06, and 50.11. Hybridizing plaques were identified and purified DNA was analyzed by restriction digestion and Southern blotting. Restriction maps and 5′ exon structures of these genes are shown in Fig. 1A. The transcription start site was assigned by primer extension analysis of olfactory RNA (data not shown). DNA sequences surrounding the putative transcriptional start sites were determined and are shown in Fig. 1B. The immediate upstream regions of these transcribed regions contained no TATA boxes and by visual and computer-assisted inspection of their sequences did not reveal extensive regions of identity. Previously isolated and sequenced cDNAs for $G_{olf\alpha}$, OcNC, and 50.06 extended to within 15 bases of the start sites predicted by primer extension. Most of the characterized cDNAs encoding type III cyclase ended approximately 50 bases short of the putative start site predicted by primer extension. One clone extended past the putative start site and matched precisely with the upstream genomic sequence. This clone could correspond to a faint signal observed in primer extension.

Binding sites in the OcNC gene. A restriction fragment from the OcNC gene containing nucleotides −312 to +154 relative to the transcription start site was used in a DNase I footprint assay. A fragment, labeled to reveal binding on the bottom strand, was incubated in the presence and absence of nuclear extracts prepared from rat olfactory tissue and subjected to limited digestion with DNase I (Fig. 2A). Two protected regions centered at positions −150 and −60 were observed. A probe labeled on the top strand of the OcNC restriction fragment was used to confirm the locations of the two protected sites.

To investigate the tissue distribution of the factors which bind to these sites, nuclear extracts from four other tissues were used in footprint assays of the OcNC promoter (Fig. 2B). The site at position −60 of OcNC (called the OcNC Olf-1 site) was protected only by extracts derived from olfactory tissue. Comparison of the sequence surrounding position −60 indicated that it was similar to the Olf-1 sites recently described (10) for the regulatory sequences of OMP, another olfactory neuron-specific gene product (11, 19). A functional role for the Olf-1-binding sequence has been suggested by experiments with transgenic mice (11, 19). The properties of the OcNC Olf-1 site and experiments presented below confirmed that the identified sequence from OcNC functions as an Olf-1-binding site. The site at −150 was completely protected by extracts from kidney and liver tissues and was weakly protected by lung extracts. This

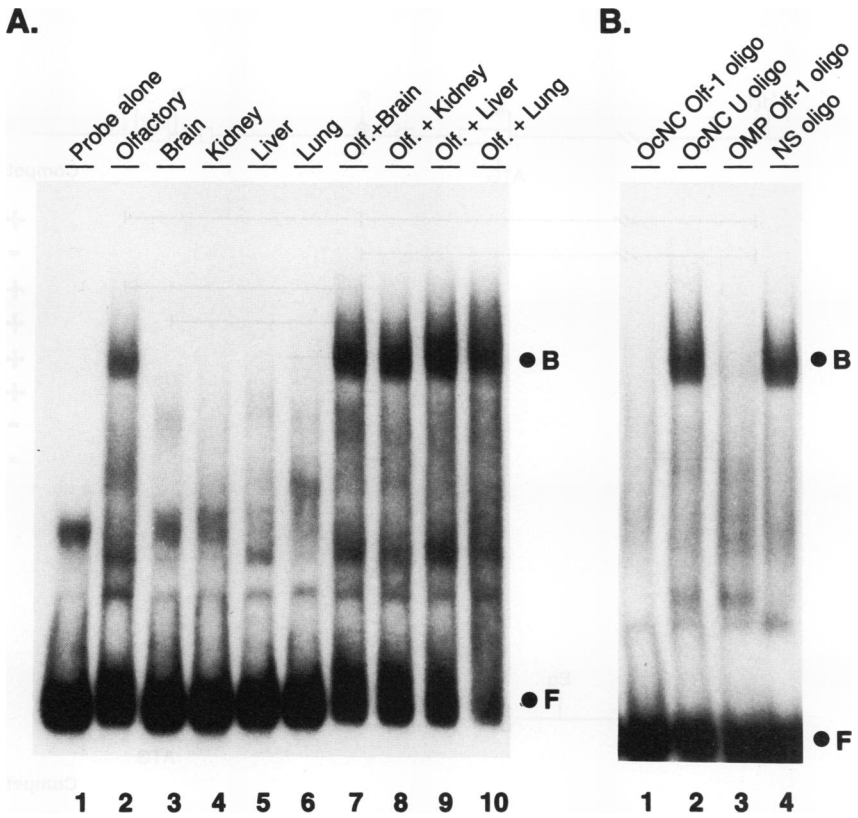


FIG. 3. Gel shift analysis of Olf-1 activity. (A) Each lane contains labeled double-stranded oligonucleotides for the OcNC Olf-1 site incubated with nuclear extracts from the indicated tissues and with mixtures of olfactory and nonolfactory tissue nuclear extracts. Nuclear extracts from testis tissue did not produce a detectable band shift (data not shown). (B) Olfactory tissue nuclear extracts were preincubated with the indicated unlabeled competitor DNAs, and then a labeled OcNC Olf-1 probe was added. NS oligonucleotides (lane 4) are unrelated to Olf-1 or U sites (top strand, 5′-GTAAGGGAGGGAGAAGCCGGTG). See Fig. 5 and 6 for sequences of other oligonucleotides. Specific DNA-protein complexes (B) and the free probe (F) are indicated.

sequence was named the U site, because it was apparently bound by a ubiquitous factor.

To further characterize the apparent olfactory tissue-specific binding activity, an end-labeled, synthetic double-stranded oligonucleotide corresponding to the OcNC Olf-1 site was used in electrophoretic mobility shift assays, a more sensitive method for demonstrating DNA-protein binding activity than footprint analysis. The OcNC Olf-1 probe formed a specific protein-DNA complex only in the presence of olfactory nuclear extracts (Fig. 3A, lanes 2 to 6). The presence of inhibitory factors in extracts from other tissues that might inhibit binding was ruled out by demonstrating that the complex formed by olfactory tissue extracts was stable in the presence of nonolfactory tissue extracts (Fig. 3A, lanes 7 to 10). The OcNC Olf-1 site probe was unable to detect Olf-1 activity in other tissues even after much longer exposures of the gel autoradiograms (data not shown). Formation of the Olf-1 complex with the OcNC Olf-1 site was inhibited by preincubation of the binding reaction with a 1,000-fold excess of unlabeled OcNC Olf-1 sites or the proximal Olf-1 site from the OMP gene (11) but was not affected by similar preincubation with a U-site oligonucleotide competitor (Fig. 3B). The Olf-1 protein-DNA complex was stable in the presence of 100 μg of poly(dI-dC) and 100 μg of salmon sperm DNA per ml and required the addition of $MgCl_2$ (not shown). The additional faint bands could not be blocked by an excess of the OcNC Olf-1 oligonucleotides and presumably reflected nonspecific complexes.

Identification of Olf-1 sites in other olfactory neuron-specific genes. The presence of Olf-1 sites in the OcNC and OMP promoter regions suggested that additional olfactory neuron-specific genes might also contain Olf-1-binding sequences. To locate functional Olf-1-binding sites in other genes, plasmids spanning overlapping regions of $G_{olf\alpha}$ and type III cyclase genomic DNA were tested for their abilities to compete for Olf-1-binding activity against labeled OcNC Olf-1 sites (Fig. 4). Briefly, overlapping restriction fragments and exonuclease III-generated truncations encompassing regions from −3 to +2.5 kb of $G_{olf\alpha}$ and from −1.1 to +0.5 kb of type III cyclase were subcloned into Bluescript and tested individually for their abilities to inhibit the formation of radiolabeled Olf-1–OcNC binding-site complexes in gel shift assays. Olf-1 sites were mapped to a 25-bp region in the second intron of $G_{olf\alpha}$ and a 150-bp region upstream of the type III cyclase transcriptional start site. To pinpoint the $G_{olf\alpha}$ and type III cyclase Olf-1 sites in these areas, oligonucleotides corresponding to 25-bp sequences which displayed the greatest similarity to the OcNC and OMP Olf-1 sites (11) were labeled and tested for their abilities to bind Olf-1 in a gel shift (Fig. 5A, lanes 2 and 7). These probes formed complexes with olfactory tissue nuclear extracts which were indistinguishable in mobility from Olf-1 complexes with

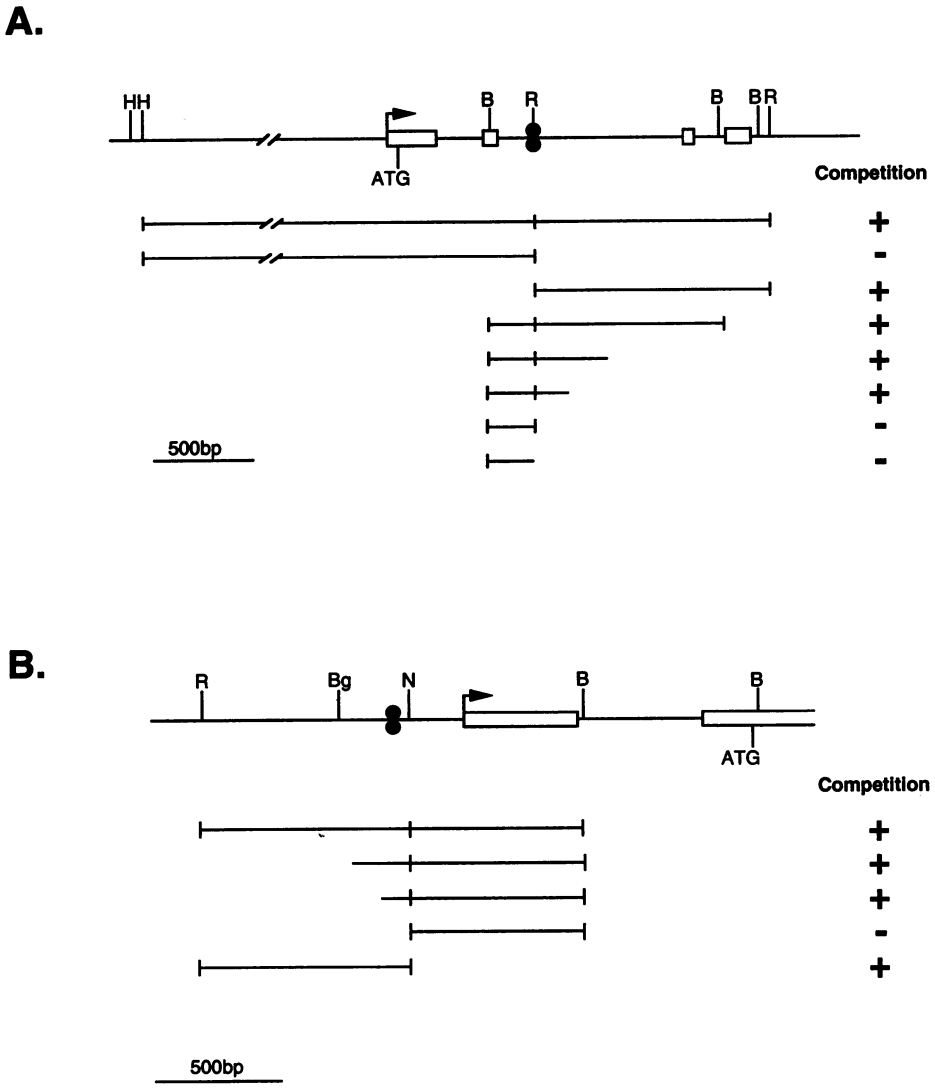
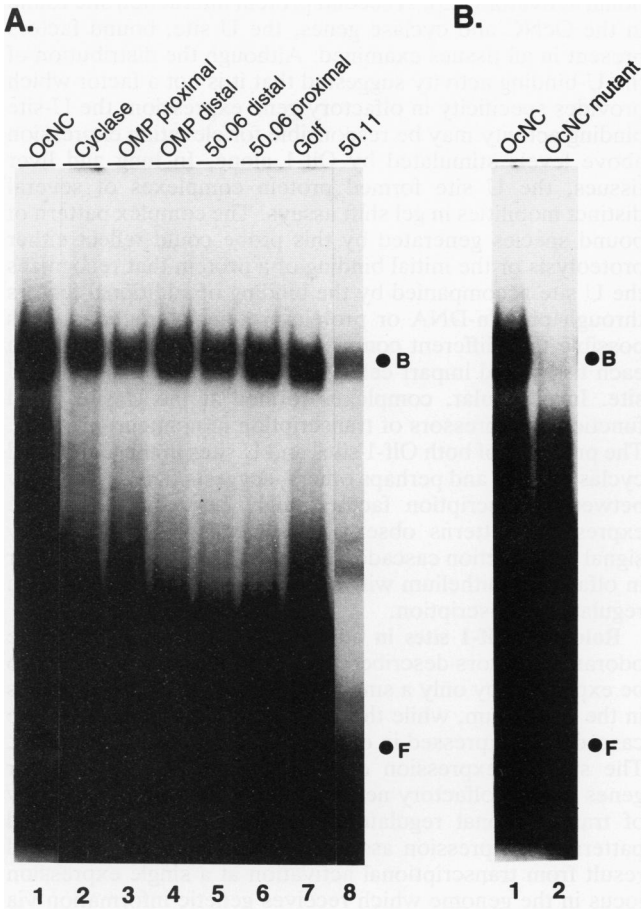


FIG. 4. Identification of Olf-1 sites in the type III cyclase and $G_{olf\alpha}$ genes. Deletion constructs spanning the $G_{olf\alpha}$ gene (A) and type III cyclase gene (B) were preincubated with olfactory tissue nuclear extracts, a labeled OcNC Olf-1 probe was added, and the complexes were analyzed by gel shift analysis. The ability of each plasmid to inhibit formation of Olf-1 probe-protein complexes is indicated on the right. A restriction map of the promoter areas of the genes (see Fig. 1 for details) is shown at the top of each deletion series. Filled circles represent Olf-1 sites. $G_{olf\alpha}$ oligonucleotides (25 bp) were synthesized for the region spanning the central *Eco*RI site. Type III cyclase Olf-1 oligonucleotides (25 bp) corresponded to a region 0.1 kb upstream of the *Eco*NI (N) site. Analysis results and the sequences of these oligonucleotides appear in Fig. 5.

OcNC (Fig. 5A, lane 1) and OMP (Fig. 5A, lanes 3 and 4) probes, and each was inhibited by incubation with unlabeled OcNC Olf-1 oligonucleotides (not shown). Additional Olf-1 sites in the olfactory neuron-specific genes 50.06 and 50.11 were identified on the basis of similarity to the sequences identified above and were found to also bind Olf-1 in gel shift assays (Fig. 5A, lanes 5, 6, and 8). The 50.11 Olf-1 site, labeled to the same specific activity as other sites, bound less well to the Olf-1-binding activity than other Olf-1 sites.

The sequences of the Olf-1 sites described here, which are summarized in Fig. 1A, and the Olf-1 sequences in OMP (11) were aligned to determine a consensus binding sequence (Fig. 5C). A mutant OcNC Olf-1 site probe containing a single C-to-G base change at a position conserved among all Olf-1 sites was tested for its ability to bind the Olf-1 protein (Fig. 5B). This mutant, changed at the second position in the consensus sequence, was unable to form a stable complex with Olf-1 in olfactory tissue nuclear extracts. Moreover, a 1,000-fold excess of the mutant failed to inhibit binding of Olf-1 to wild-type Olf-1 probes (data not shown).

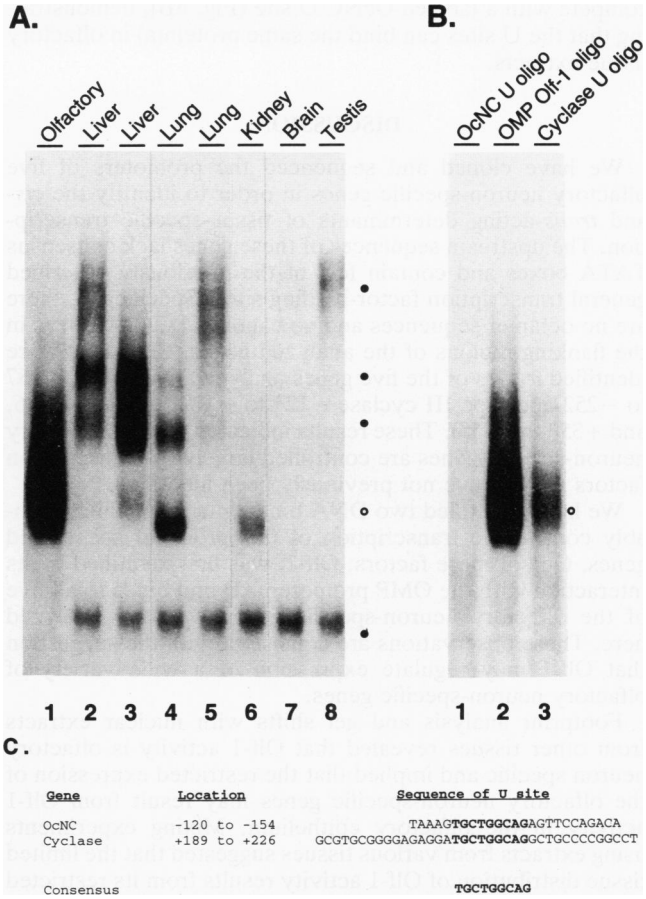
Analysis of the U site. DNase I footprint analysis revealed that the OcNC U site was bound by factors present in several tissues. To further characterize this binding activity, gel shift analysis was performed on nuclear extracts from six tissues by using a labeled OcNC U-site probe (Fig. 6A). By using extracts derived from olfactory tissue, a complex which was similar in mobility to complexes formed from liver, lung, and kidney extracts was detected (Fig. 6A, lanes 1 to 6). Extracts from liver and lung tissues formed additional specific complexes of distinct mobilities which varied in abundance among extract preparations. Brain and testis extracts reproducibly formed very-low-mobility complexes



C.

Gene	Location	Sequence of Olf-1 site
OcNC	-72 to -39	AAGGGAGA**GTCCCTAGGGAG**CTTGGGAGGGGCCA
Cyclase	-281 to -257	CTGTATA**TTCCCTTGAGGA**GAGATC
Golf	approx. +750	AAGACCAA**TTCCCCCAAGAA**TTCCT
50.06 distal	-97 to -73	TCTCAGGA**TTCCCCAGGGAG**GGGAC
50.06 proximal	+759 to +735	AGCAA**CTCCCTGGAGGC**TGTGGGAT
OMP distal	-710 to -686	TCACAT**CTCCCAGGGGAG**GTGGAGG
OMP proximal	-186 to -162	GCTCTG**GTCCCCAAGGAG**CCTGTCA
50.11	-91 to -68	TTACTA**CTCCTCAGGGTA**TATGCC
Consensus		YTCCCYRGGGAR
OcNC mutant		AAGGGAGA**GTGCCTAGGGAG**CTTGGGAGGGGCCA

FIG. 5. Binding of Olf-1 to sites from five olfactory neuron-specific genes and mutational analysis. (A) Each lane shows gel shift analysis of olfactory tissue nuclear extracts incubated with labeled oligonucleotide probes encoding Olf-1 sites from the five genes analyzed and OMP. Sequences of the oligonucleotide probes are displayed in panel C. Positions of bound (B) and free (F) probes are indicated. (B) Wild-type or mutant oligonucleotides which contain a single base pair substitution in the OcNC Olf-1 site were labeled and tested for their abilities to bind Olf-1 from olfactory tissue nuclear extracts by using gel shift analysis. (C) Oligonucleotide probes for Olf-1 sites (boldface), their locations within the olfactory neuron-specific genes, and alignment of their sequences. The consensus for the Olf-1-binding sites represents bases that appear in more than half of the sites. Y = C or T; R = G or A. All sequences indicated, except for the 50.06 proximal and $G_{olf\alpha}$ oligonucleotides, represent the top strand of the genomic sequence. All probes used for binding assays were double stranded. The precise distance between the $G_{olf\alpha}$ Olf-1 sites and the start site has not been determined by sequencing on both strands.



C.

Gene	Location	Sequence of U site
OcNC	-120 to -154	TAAAG**TGCTGGCAG**AGTTCCAGACA
Cyclase	+189 to +226	GCGTGCGGGGAGAGGA**TGCTGGCAG**GCTGCCCCGGCCT
Consensus		TGCTGGCAG

FIG. 6. Gel shift analysis of U-site binding activity. (A) Each lane contains labeled double-stranded oligonucleotides for the OcNC U site incubated with nuclear extracts from the indicated tissues. Different preparations of a tissue extract are shown for comparison (compare lanes 2 and 3 and lanes 4 and 5). Open circle, position of the complex formed with olfactory tissue extracts; filled circles, positions of complexes formed with extracts of other tissues. The probe was run off the gel. (B) Olfactory tissue nuclear extracts were preincubated with unlabeled specific competitor DNA encoding the OcNC U site (lane 1), the OMP proximal Olf-1 site (lane 2), or the type III cyclase U site (lane 3), and then a labeled OcNC U-site probe was added. (C) Oligonucleotide probes for U sites, their locations in the olfactory neuron-specific genes, and alignment of their sequences. The area of identity between the sequences is boldfaced. The type III cyclase sequence and the OcNC sequence are top and bottom strand sequences, respectively.

(Fig. 6A, lanes 7 and 8). Each of these complexes can be specifically blocked with an unlabeled probe (data not shown).

Footprint analysis of the 5′ untranslated region of the type III cyclase gene identified a region at +200 which was protected by a binding activity present in olfactory tissue nuclear extracts (data not shown). The sequence, called the cyclase U site, corresponding to this footprint was identical to the OcNC U site over 9 bases (Fig. 6C). To determine whether the OcNC U site and the cyclase U site bound the same protein in olfactory tissue extracts, an excess of cyclase U oligonucleotide was tested for its ability to compete for the U-binding factor in a gel shift assay. Unlabeled oligonucleotides encoding the OcNC U site and the cyclase U site, but not the Olf-1 site, DNA were able to specifically

compete with a labeled OcNC U site (Fig. 6B), demonstrating that the U sites can bind the same protein(s) in olfactory tissue extracts.

DISCUSSION

We have cloned and sequenced the promoters of five olfactory neuron-specific genes in order to identify the *cis*- and *trans*-acting determinants of tissue-specific transcription. The upstream sequences of these genes lack consensus TATA boxes and contain few of the previously described general transcription factor-binding sites. Specifically, there are no octamer sequences and no canonical CAAT boxes in the flanking regions of the analyzed genes. SP-1 sites were identified in two of the five genes analyzed here ($G_{olf\alpha}$ −257 to −252 and type III cyclase −127 to −122, +111 to +116, and +350 to +355). These results indicated that the olfactory neuron-specific genes are controlled largely by transcription factors which have not previously been identified.

We have identified two DNA binding factors which probably control the transcription of this group of specialized genes. One of these factors, Olf-1, was first identified by its interaction with the OMP promoter (11) and binds to all five of the olfactory neuron-specific genes we have analyzed here. These observations are consistent with the suggestion that Olf-1 may regulate expression of a wide variety of olfactory neuron-specific genes.

Footprint analysis and gel shifts with nuclear extracts from other tissues revealed that Olf-1 activity is olfactory neuron specific and implied that the restricted expression of the olfactory neuron-specific genes may result from Olf-1 activity in the olfactory epithelium. Mixing experiments using extracts from various tissues suggested that the limited tissue distribution of Olf-1 activity results from its restricted presence in olfactory tissue rather than the presence of inhibitory components in nonolfactory cell types. A functional role of the Olf-1 site in the OMP promoter has been suggested by Kudryki et al. (11), who have demonstrated that a 0.3-kb fragment of the OMP promoter containing one Olf-1 site is sufficient to drive olfactory neuron-specific transcription of a reporter gene in transgenic mice. In sum, these results suggest a model in which Olf-1, which is itself confined to the olfactory neurons and whose presence developmentally precedes expression of mature olfactory markers (11), functions as a critical transcription factor which orchestrates the activation of olfactory neuron-specific genes. Analysis of a cDNA that encodes the Olf-1-binding activity confirms this model (21). The cloned Olf-1 protein, when expressed in mammalian cells, functions as a transcriptional activator of synthetic promoters containing Olf-1 sites. Moreover, of the six tissues tested, Olf-1 mRNA and activity are found only in olfactory epithelium. Finally, immunohistochemical analysis of the Olf-1 protein demonstrates that both mature neurons and relatively immature precursor cells express Olf-1.

Properties of the Olf-1 protein can be predicted from inspection of the consensus DNA binding site derived from the eight identified Olf-1 sites (YTCCCYRGGGAR). Point mutations of conserved residues within the consensus abolish binding (Fig. 5B) or lower binding affinity (e.g., the 50.11 Olf-1 site), indicating that Olf-1 binds very specifically to its recognition site and that such sites occur by chance very infrequently ($<1/10^6$ bp). Further, the dyad symmetry of the Olf-1 site suggests that the Olf-1 protein may bind to DNA as a homodimer.

Specific transcription in other systems arises from the combined activities of tissue-specific and general transcriptional activators (15). A second protein interaction site found in the OcNC and cyclase genes, the U site, bound factors present in all tissues examined. Although the distribution of the U-binding activity suggested that it is not a factor which provides specificity in olfactory gene expression, the U-site binding activity may be responsible for elevating expression above levels stimulated by Olf-1 alone. In lung and liver tissues, the U site formed protein complexes of several distinct mobilities in gel shift assays. The complex pattern of bound species generated by this probe could reflect either proteolysis or the initial binding of a protein that recognizes the U site accompanied by the binding of additional factors through protein-DNA or protein-protein interactions. It is possible that different complexes assemble at the U site in each tissue and impart cell-type-specific functions to the U site. In particular, complexes formed at the U site could function as repressors of transcription in nonneuronal cells. The presence of both Olf-1 sites and U sites in the OcNC and cyclase genes, and perhaps others, suggests that an interplay between transcription factors might be required for the expression patterns observed for genes in the olfactory signal transduction cascade. Characterization of the U factor in olfactory epithelium will more precisely define its role in regulating transcription.

Roles for Olf-1 sites in odorant receptor expression. The odorant receptors described by Buck and Axel (3) appear to be expressed by only a small fraction of the mature neurons in the epithelium, while the downstream components of the cascade are expressed in every olfactory neuron (2, 10, 16). The specific expression of a limited number of receptor genes in each olfactory neuron could arise from a hierarchy of transcriptional regulators. Alternatively, the restricted patterns of expression associated with the receptors could result from transcriptional activation at a single expression locus in the genome which receives genetic information via gene conversion from one of the hundreds (3, 12) of transcriptionally silent receptor loci. In this model, the presence of Olf-1-binding sites at the expression locus could direct olfactory neuron-specific transcription at that location and permit the limited expression of a particular receptor while the remaining components of the cascade are expressed uniformly in the olfactory neurons of the epithelium. While the mechanism which regulates specific receptor expression remains unknown, sites similar to those described here may be targets for factors directing expression of odorant receptor genes by the sensory neurons.

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